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14. ABSTRACT

Silk fibers formed by insects and spiders are noted for their remarkable mechanical properties as well as their durability and biocompatibility. The exceptional solubility *in vivo* (20-30% w/v) of these proteins is dictated by both the need to produce solid fibers with a high packing fraction and the high mesogen concentration required for lyotropic liquid crystalline spinning, while also achieving high end mechanical properties for survival (orb webs, cocoons). Combining knowledge of the solution state behavior, protein folding requirements and silk genetic/protein designs employing complex block-copolymer attributes, offers new experimental directions. Our *objective* was to determine the relationships between genetic/protein block designs coupled with the limitations imposed by an all aqueous processing environment. The *significance* of the studies was that by employing these design rules there should be improved expression, recovery of soluble protein and control of processing into high solids solutions and gels leading to spinnable dopes for fibers, films or other material outcomes. The insights from the studies have implications in fundamental structural biology as well as direct utility toward improved options in silk-based polymer synthesis, processing and materials fabrication.

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Subject: Final Report (Short Form) - *Coupling Silk Expression & Processing by Genetic Design*

Principle Investigator: *David Kaplan, Tufts University*

Summary:
Silk fibers formed by insects and spiders are noted for their remarkable mechanical properties as well as their durability and biocompatibility. The exceptional solubility *in vivo* (20-30% w/v) of these proteins is dictated by both the need to produce solid fibers with a high packing fraction and the high mesogen concentration required for lyotropic liquid crystalline spinning, while also achieving high end mechanical properties for survival (orb webs, cocoons). Combining knowledge of the solution state behavior, protein folding requirements and silk genetic/protein designs employing complex block-copolymer attributes, offers new experimental directions in the construction, expression and assembly of silk proteins that can help generate new and useful silk-based materials for a range of functions. Our *objective* was to determine the relationships between genetic/protein block designs coupled with the limitations imposed by an all aqueous processing environment based on our recent models of how silk proteins are assembled in solution toward gel states and then spinning. The *significance* of the studies was that by employing these design rules there should be improved expression, recovery of soluble protein and control of processing into high solids solutions and gels leading to spinnable dopes for fibers, films or other material outcomes. The insights from the studies have implications in fundamental structural biology as well as direct utility toward improved options in silk-based polymer synthesis, processing and materials fabrication. During the program we focused on new modes of silk modification, assembly, characterization and materials properties to expand the options with this unique family of proteins.

As examples, we have developed:

- processes for self-standing thin films of silk and determined mechanical properties for these materials, indicating the unique functional attributes of these protein systems - these results have implications in light-weight conformal materials designs. Remarkably robust mechanical stability with 100 nm thick films was demonstrated.
- new modes to functionalize silks, through specific chemical modifications to generate pegylated systems to control surface interactions – this approach alters water interactions at the surface and can be used as a mode to regulate the adsorption of other molecules or the interaction of cells with these surfaces.
- silk-inorganic interfaces through controlled mineralization with silica and hydroxyapatite via genetic engineering approaches – these studies demonstrated the ability to functionalize silks in new ways to integrate inorganic components directly with the protein material, the hydrophobic nature of silk excludes the additional chimeric fusions responsible for inorganic nucleation and thus providing a novel way to utilize interfacial material assembly to control the mineralization phase of these new materials.
- further elucidated the mechanistic basis for silk gelation, leading to new modes to control this process and expand the utility of these novel forms of silk – these advances allow gelation to occur in more rapid fashion than the slower self-assembly process, greatly expanding the potential applicability of these systems for new material needs.
- novel optical systems based on silk materials that offer a new technology platform in sensors and related areas – the ability to generate optically clear yet robust films from silk using the all-aqueous process developed in the program, allows for the incorporation of biological components in the film to establish new optical platforms based on the combination of biological selectivity and the material properties of silks.

In total, the above systems were realized through the fundamental insight into the role of hydrophobic hydration on the process of silk assembly. This has opened new venues to exploit this protein biomaterial in areas never previously considered – such as optics, gel delivery platforms and robust thin films, as just some examples. The DoD impact of the work is anticipated to be in areas of high performance materials and nanocomposites, as well as in tough light-weight conformal coatings, distributed biosensors and related needs.

Publications from the Grant:

Mapping domain structures in silks from insects and spiders related to protein assembly. Bini, E., D. Knight, D. L. Kaplan. *J. Molecular Biology*. 335:27-40 (2004)

Water-Stable Silk Films with Reduced β-Sheet Content, Jin, H. J., J. Park, V. Karageorgiou, U. J. Kim, R. Valluzzi, P. Cebe, D. L. Kaplan, *Advanced Functional Materials*, 15:1241-1247 (2005).

Three dimensional aqueous-derived biomaterial scaffolds from silk fibroin. Kim, U.J., Park, J., Kim, H.J., Wada, M. and Kaplan, D.L. *Biomaterials*, 26: 2775-2785, (2005)

Biomaterial coatings by stepwise deposition of silk fibroin. Wang, X., Kim, H. J., Xu, P., Matsumoto, A. and Kaplan, D. L. *Langmuir* 21(24): 11335-11341, (2005).

Role of pH and charge on silk protein assembly in insects and spiders. Wong C., E. Bini, J. Huang, S. Y. Lee, D. L. Kaplan. *Applied Physics A: Materials Science and Processing*, 82:223-233 (2006).

Solution behavior of synthetic silk peptides and modified recombinant silk proteins. Wong, C., E. Bini, J. Henseman, D. P. Knight, R. V. Lewis, D. L. Kaplan. *Applied Physics A: Materials Science and Processing*, 82:293-203, (2006).

Unfolding the multi-length scale domain structure of silk fibroin protein. H. Shulha, C. Wong, D. L. Kaplan, V. V. Tsukruk. *Polymer* 47:5821-5830 (2006).

Production of submicron diameter silk fibers under benign processing conditions by two-fluid electrospinning. M. Wang, J. H. Yu, D. L. Kaplan, G. C. Rutledge. *Macromolecules*, 39: 1102-1107 (2006).

Novel nanocomposites from spider silk-silica fusion (chimeric) proteins. Wong, C. P. F., Kitchel, B., Huang, J., Patwardhan, S.V., Belton, D., Perry, C., Kaplan, D. L. *Proc. National Academy of Science USA* 103(25): 9428-9433 (2006).

Covalently immobilized enzyme gradients within three-dimensional porous scaffolds. Vepari, C. and Kaplan, D.L. *Biotechnology and Bioengineering*, 93:1130-1137 (2006)

Fibrous Proteins – Role in Biomaterials and Tissue Engineering. Wang, X., Kim, H.-J., Wong, C., Vepari, C., Matsumoto, A. and Kaplan, D.L. *Materials Today* 9:44-53, (2006)

Mechanisms of silk fibroin sol-gel transitions. A. Matsumoto, J. Chen, A. L. Collette, U.J. Kim, G. H. Altman, P. Cebe, D. L. Kaplan. *J. Physical Chem*. In press (2006).

Effect of water on the thermal properties of silk fibroin. X. Hu, D. L. Kaplan, P. Cebe. *Thermochimica Acta*. In press (2007).

RGD-Functionalized Bioengineered Spider Dragline Silk Biomaterial. E. Bini, C. Wong, J. Huang, V. Karageorgiou, B. Kitchel, D. L. Kaplan *Biomacromolecules*, *7:3139-3145* (2007)

Bioengineered Spider Silk-Dentin Matrix Protein 1 Chimeric Protein Induces Hydroxyapatite Nucleation. J. Huang, C. Wong, A. George, D. L. Kaplan *Biomaterials*. 28:2358-2367 (2007)

Additional Details on Some Findings:

Control of Silk Gelation – Silk fibroin sol-gel transitions were studied by monitoring the process under biologically relevant physicochemical conditions using optical spectroscopy. The secondary structural change of the fibroin from a disordered state in solution to a beta-sheet-rich conformation in the gel state was assessed by FTIR and CD over a range of fibroin concentrations, temperatures and pHs. Structural changes were correlated to gelation based on changes in optical density at 550 nm. No detectable changes in the protein secondary structure (FTIR, CD) were found up to about 15% gelation, indicating that these early stages of gelation are not accompanied by the formation of beta sheets, while above 15%, the fraction of beta sheet increased linearly with degree of gelation. A pH dependency of gelation time was found with correlation to the predominant acidic side chains in the silk. Electrostatic interactions were related to rate of gelation above neutral pH. The overall independencies of processing parameters including silk fibroin concentration, temperature and pH on gel formation and protein structure can be related to primary sequence-specific features in the molecular organization of the fibroin protein. These findings clarify aspects of the self-assembly of this unique family of proteins as a route to gain control of material properties, as well as for new insight into the design of synthetic silk-biomimetic polymers with predictable solution and assembly properties.

Versatility in Processing and Functionalization – Silk proteins can be reprocessed into a wide range of material morphologies (e.g., nanodiameter fibers, porous spongy matrices, hydrogels, thick fibers, films, blocks) and structures (high or low beta sheet content) depending on the mode of solution preparation. We have extensive experience with formation of a range of material morphologies generated from silk proteins, including films, fibers, hydrogels and 3D porous matrices.

Fibers – We have generated nanoscale diameter fibers via electrospinning and studied nanomechanical properties using AFM nanoindentation techniques. The biological compatibility of these materials in terms of adhesion and growth of human bone marrow derived stromal cells was also reported (Jin et al., 2004). In addition, we have conducted two fluid electrospinning to generate silk-based tubular systems amenable to loading of additional materials.

Films - Films have been generated from reprocessed silk fibroin in a variety of modes to control structure and morphology (Jin et al., 2004; 2005). In addition, these films have been chemically modified to study cell adherence and differentiation toward tissue specific outcomes such as bone (Sofia et al., 2001; Chen et al., 2003; Karageorgiou et al., 2005).

Hydrogels – Apart from the mechanistic studies mentioned earlier, hydrogels were formed from aqueous silk solutions through osmotic stress, leading to control of gel features based on the concentration of silk protein, the concentration of the osmotic stress inducing polymer (high molecular weight polyethylene glycol, PEG), pH, temperature and the presence of divalent or monovalent cations (Kim et al., 2005a; 2005b).

3D Porous Matrices - Porous 3D scaffolds were formed from regenerated silk fibroin using freeze-drying, salt leaching and gas foaming techniques with porosities up to 99% and pore sizes controllable from 10s of microns to 1,000 microns depending on processing details (Nazarov *et al.*, 2004; Kim et al., 2005a).

Control of Structure and Morphology – Nanometer Length Scales - Ultrathin Silk Fibroin Coatings - In our recent studies we have elucidated self-assembly pathways for these proteins (Jin and Kaplan, 2003) and the role of the specific domains in protein folding leading to fiber formation (Bini et al., 2004; Wong et al., 2005). We have recently extended these observations to controlled deposition of ultrathin layers (nanometer length scale) by exploiting our fundamental understanding of the self-assembly of this protein and solution control of protein assembly (Wang et al., 2005) (**Figure 3**). The approach proposed is unique in that it is a completely aqueous, stepwise deposition process, and the structural control of the silk protein locks in the features of the coatings due to the physical cross-links (beta sheets). To validate the successful stepwise nanocoat deposition process, a detailed physical

characterization study of the deposited layers was conducted (Wang et al., 2005). In a representative procedure, a cleaned substrate (gold electrode, quartz or hydrophobically modified glass) was immersed in a silk aqueous solution (1 mg/ml) for 2 min at RT and subsequently washed with deionized water for 1 min, then dried with dry nitrogen gas for 2 min. This process was repeated until the desired number of layers was assembled. UV-Vis spectroscopy and research quartz crystal microbalance (RQCM) were used to monitor the deposition. The multilayer adsorption processes were linear and reproducible (**Figure 3**). The layer thickness could be controlled by varying the concentration of silk fibroin solution (0.1 to 4.0 mg/ml), pH (2.5 to 12.5) or salts (0 to 1.0 M NaCl). For example, as the concentration of NaCl increased, the single layer thickness increased from 7.4 nm to 13 nm. The preliminary studies of nanocoat structure by ATR-FTIR showed one strong peak at 1622 cm^{-1}, characteristic for antiparallel β-sheet structures, with up to 47% beta sheet content in the materials. The formation of this beta sheet silk structure (termed silk II) was induced by nitrogen gas, likely due to dehydration effects, as is observed with MeOH treatment. The deposition was also affected by the pH of the solution when a charged substrate was used. Using substrates with different hydrophobicity, the deposition could be manipulated by varying the collective contribution of both hydrophobic and electrostatic interactions.

Silk-Inorganic Nanocomposites – Silica Systems – The basis for Aim #1 of the present proposal originates from our recent studies in collaboration with C. Perry and R. Naik on the construction, cloning, expression and function of silk-R5 fusions for formation of silica nanocomposites. Functionalized silk protein was fused with R5 peptide (Wong et al., 2006). In this preliminary study, we cloned oligonucleotides encoding the R5 peptide unit of Sil1 protein to a 15mer spider silk dragline silk (*N. clavipes*, major ampullate gland silk 1, consensus repeat), and expressed this fusion protein. The purified protein was formed into films and electrospun fibers and used as substrates to conduct silicification reactions. The structural variants generated included: (a) silk domain (15mer) with R5: 15mer+R5 (with and w/o the CRGD), and (b) silk domain (15 mer) without R5: 15mer (with and w/o the CRGD). The amino acid composition of the synthetic spider silk fusion proteins agreed with the expected composition. Yields of purified recombinant fusion proteins were approximately 100-150 mg/L. The CRGD15mer+R5 protein had an apparent molecular weight of approximately 45 kDa, in agreement with the expected molecular weight of 46.5 kDa. The 15mer+R5 recombinant protein also showed an apparent molecular weight of approximately 45 kDa, consistent to the calculated molecular weight of 45.2 kDa. SEM analysis indicated that the genetically engineered spider silk proteins fused with R5 formed silica precipitates with diameters up to ~1,000 to 2,000 nanometers (**Figure 4**). Control silicification reactions were also run using the genetically engineered spider silk proteins and no silica precipitates were observed. The results from silica formation reactions from films cast from the genetically protein variants, wherein films were first formed (from water or methanol) and then silica reactions conducted with 1 M TMOS in 1 mM HCl in 100 mM phosphate buffer at pH 5.5. Additional controls were included as well to confirm the selective response related to the presence of R5 in the sequences. Importantly, the difference in morphology on the silk films prepared by two different processing methods provides additional future options for control of morphology.